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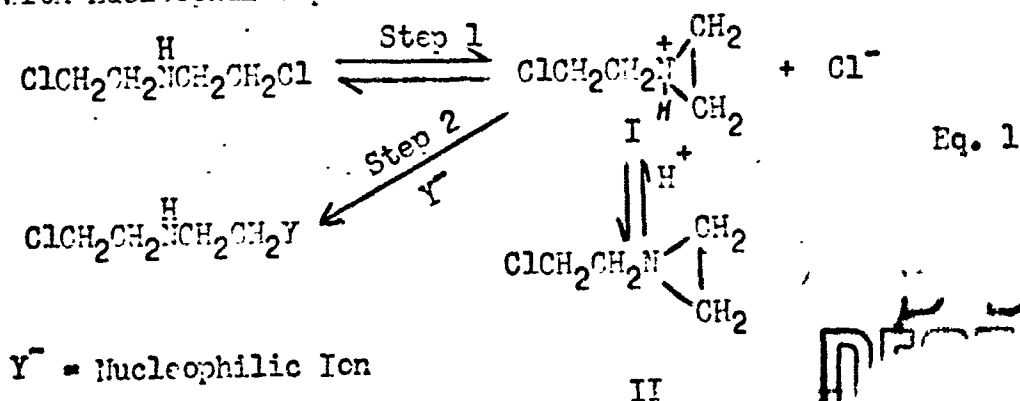
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THE ENZYMIC TRANSFORMATION OF SECONDARY NITROGEN
MUSTARDS: IMPLICATIONS IN RESPIRATION

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Since their introduction as potential chemical warfare agents, the nitrogen mustards have served as valuable research tools for studies of the vital reproductive processes, as well as other basic biologic mechanisms. Although widely employed to achieve such effects as mutations, radiation-type symptoms and carcinogenesis, the most rewarding applications of these agents have been in the field of cancer chemotherapy. A broader understanding of the *in vivo* mode of action of the nitrogen mustards would contribute significantly to improved design of compounds for both military and civilian uses. The biologic effects of these drugs are generally ascribed to chemical alkylation of susceptible functions such as sulfhydryl, amino, and ring nitrogen of purines and phosphate (e.g., Refs. 1,2, 3).

The secondary nitrogen mustard, N,N-bis(2-chloroethyl)amine (nor-IM2) was selected for a detailed study of its reaction mechanism. Previous work (4) indicates that in aqueous media it reacts via an aziridinium ion I (Equation 1). It is this ion that reacts with nucleophiles present in solution.

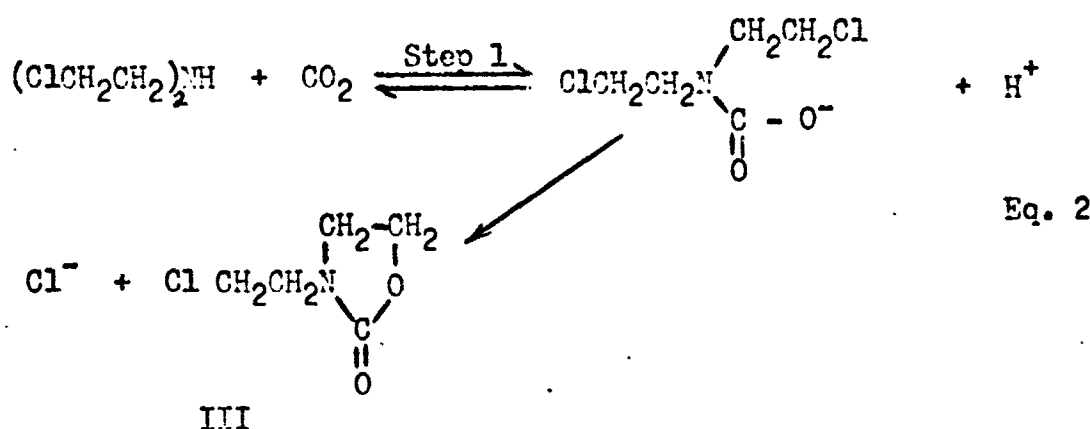


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The protonated aziridinium ion I alkylates nucleophilic substances considerably faster than the uncharged species II which is largely present in basic media (4).

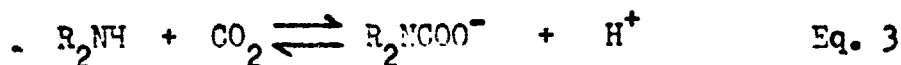
For this reason nor-MN2 and other secondary amine mustards are generally more reactive in solutions of low pH, where the concentration of the protonated form is high. Two well known secondary nitrogen mustards of this type that have been used extensively in cancer chemotherapeutic studies are nor-MN2, and 1,6-di-(2-chloroethyl-amino)-1,6-deoxy-D-mannitol (mannitol mustard, degranol). The pK_a 's of these compounds (pK_a of 6.5 for Nor-MN2 and 7.2 for Degranol) are such that the aziridine formed on cyclization exists to a considerable extent in the unprotonated form at physiologic pH. Thus, they react quite slowly when dissolved in an aqueous solution of pH 7.4 (4).

Both nor-MN2 and degranol disappeared from human and animal blood serum much more rapidly than might be predicted on a theoretical basis. This unusually rapid disappearance can be interpreted in terms of a newly reported reaction between nor-MN2 and carbon dioxide to form 3-(2-chloroethyl)-2-oxazolidinone (5,6) (Equation 2). The reaction has been noted to proceed spontaneously in aqueous sodium bicarbonate solution.



The studies with nor-MN2 reported herein demonstrate the presence of a hitherto unknown enzyme in human and animal blood serum that catalyzes formation of the oxazolidinone. Thus, after injection into the bloodstream, these agents are rapidly transformed into reaction products that cannot perform the alkylating reaction so characteristic of the nitrogen mustards.

The kinetic data indicate that this enzyme catalyzes carbonate formation - a reaction of importance for the transport of carbon dioxide in blood.



Reexamination of the presently accepted mechanism of normal respiration suggests that those amines in blood that are capable of transporting CO_2 via carbonates exhibit low pK_a values and react rather slowly with CO_2 , as compared with more basic amines. Since, in the lungs, blood contacts a lowered partial pressure of CO_2 for only a brief period, this non-catalyzed method of CO_2 transfer would be expected to be ineffective. It is unlikely that the *in vivo* action of this enzyme is limited to 2-chloroethylamines. More probably, it operates with naturally occurring amines as well, thereby catalyzing the reaction shown in equation 3, and serving an important function of carbon dioxide transport in the body.

A. Nature of the Reaction Between Nor-HN2 and Blood

The rate of cyclization of nor-HN2 in 0.1 M. pH 7.4 phosphate buffer, 37° (step 1, eq. 1) was measured by liberation of chloride ion, and the first order velocity constant was found to be $3 \times 10^{-2} \text{ min}^{-1}$ (half life = 23 min.). In this system, the cyclized form of nor-HN2 is stable for many hours. In fresh human blood, however, nor-HN2 reacted more rapidly than would be predicted. In spite of its rather slow cyclization rate, 50% of the initial concentration disappeared in approximately 2 minutes.^a

The typical reaction patterns of nor-HN2 in various concentrations of fresh rabbit blood are shown in Fig. 1. These curves illustrate two outstanding features of the reaction between nor-HN2 and blood: (a) the decrease in reaction rate with decrease in concentration of blood and (b) the obvious retardation of the reaction rate in its later stages as well as the fact that it does not proceed to completion.

The 4-(4'-nitrobenzyl)pyridine test^b used for the determination of the percentage of alkylating agent remaining at various time intervals measures both the uncyclized and cyclized forms of nor-HN2 (Eq. 1). The alkylating activity remaining in the final stages of the reaction in Fig. 1 could be one or both of these species. Each curve in Fig. 1, therefore, describes two reactions occurring simultaneously in blood: (a) a rapid reaction that destroys the nor-HN2, and (b) the formation of a second alkylating

^a The time required for disappearance of half of the agent varied from less than 1 min. to several min. depending on the particular blood sample used.

^b The NBP method (7) was adapted for these rate studies to determine the amount of nor-HN2 remaining at any particular time (8). The rate of cyclization of nor-HN2 in 0.1 M phosphate buffer was determined by titration of liberated chloride ion, using a modification of the mercuric-diphenylcarbazone method (9).

agent which is not destroyed by the active principle in blood. Furthermore, the quantity of the latter appears to be dependent on the overall rate of reaction. Thus in Fig. 1, the slower reaction produces the greater quantity of alkylating agent in a form unreactive toward blood.

The reaction pattern set forth in Fig. 1 suggests a rapid reaction between uncyclized nor-HN2 and blood.^c This is most probably the reaction shown in equation 2 in which carbon dioxide is responsible for the rapid reaction. To account for the alkylating agent remaining in blood, it is postulated that this is the cyclized nor-HN2 (II). The cyclization process (Eq. 1), which begins immediately when nor-HN2 is dissolved in blood, leads to the formation of cyclized nor-HN2 (II) that would normally be expected to react only very slowly with blood.

To test this hypothesis, a series of experiments were performed to determine the stability of the cyclized form of nor-HN2 (II) in fresh blood. In these experiments nor-HN2 was incubated in pH 7.4 buffer to yield various percentages of II which were then allowed to react with fresh goat blood. Specifically, nor-HN2 (50 μ g./ml.) was dissolved in 0.1 M, pH 7.4 phosphate buffer maintained at 37°, and cyclization (Eq. 1) was allowed to proceed. Periodically, aliquots (containing various percentages of cyclized product) were removed and allowed to react with fresh goat blood. As shown in Fig. 2, the cyclized form of nor-HN2 is essentially unreactive towards fresh goat blood. The experimental curve depicted in Fig. 2 exhibits a half-life of cyclization of approximately 23 min. This value agrees with the cyclization rate as measured by the rate of chloride ion liberation. It may, therefore, be concluded that any cyclized nor-HN2 present in Fig. 1 will also be relatively unreactive.

^c Theoretically, the reaction proceeds stepwise. In accordance with equation 1, step 1 (cyclization) begins upon contact with water, and nucleophilic ions (step 2) then act upon the protonated cyclized form. For aliphatic nitrogen mustards, in general, step 1 is reasonably fast while step 2 is slow and is, therefore, the rate controlling step in the overall process. The cyclic species accumulates to relatively high concentrations in the reaction media even in the presence of the highly nucleophilic thiosulfate ion (IO). The uncyclized form of nor-HN2 is a poor alkylating agent as compared to the cyclized form and the overall reaction rate as depicted in equation 1 should never exceed the rate of cyclization (step 1). Because the reaction of nor-HN2 in fresh blood occurs at a much faster rate than step 1, it obviously does not proceed through the cyclic intermediate ion I and, therefore, is not the normal alkylation reaction.

As a corollary, the quantity of unreactive alkylating agent remaining in solution after reaction with blood is inversely proportional to the overall reaction rate of nor-HN2 in blood. Accordingly, the quantity of alkylating agent remaining after reaction with blood under standard conditions was utilized as an inverse index of reactivity of nor-HN2 in blood. It is noteworthy that this remaining alkylating agent showed no measurable decline after several hours in blood.

To evaluate the role of carbon dioxide, the rate of the reaction shown in equation 2 was measured in the absence of blood; i.e., in sodium bicarbonate solution strongly buffered with phosphate buffer at pH 7.4. Fig. 3 is a plot of the pseudo first order velocity constants of nor-HN2 at various concentrations of sodium bicarbonate in 0.25 M, pH 7.4 phosphate buffer at 37°. In Table 1 are tabulated similar values when nor-HN2 is allowed to react with various biologic media under identical conditions. The large deviations between species are outstanding and incompatible with the predicted course of equation 2 in animal blood. The data in Table 1 shows that a concentration of approximately 0.2 M sodium bicarbonate is required to obtain a reaction rate of the same velocity as obtained in fresh guinea pig blood whereas only approximately 0.02 M. sodium bicarbonate is required to obtain the same reaction rate as nor-HN2 in mouse blood.

If these deviations in reaction rates of nor-HN2 are to be ascribed solely to the concentration of dissolved carbon dioxide (e.g., free CO₂, bicarbonate or carbonates), the quantities of CO₂ in guinea pig blood must be approximately ten fold that in mouse blood. The normal concentrations of total dissolved carbon dioxide in mammalian blood is approximately 0.025 M. (11). Deviations as low as 0.015 M. have been observed in diabetic coma and nephritic death (12). Deviation as high as 0.025 M. has been obtained when large quantities of sodium bicarbonate were experimentally administered to laboratory animals (12). The differences observed in the reaction rates of nor-HN2 in mammalian blood cannot, therefore, be attributed solely to a change in blood CO₂ concentration. Although the reaction mechanism for the disappearance of nor-HN2 may be that illustrated in equation 2, the rate at which this process occurs in blood appears to be dependent upon factors other than the total quantity of carbon dioxide present. It should be noted that mouse blood does not possess the ability to increase the rate of the reaction depicted by equation 2 beyond that shown in Fig. 3.

Table 1

Reaction Rate of Nor-HN₂ in Various Biological Media

Biological Medium	Half Life, min. ^a		Molarity of Bicarbonate Required to Produce Similar Rate ^d
	Biological Medium ^b 25%	Biological Medium ^c 100%	
Human, Whole Blood	9.3	2.4	0.042
Human, Serum	8	2.0	0.05
Guinea Pig, Whole Blood	2.0	0.5	0.2
Mouse, Whole Blood	18.5	4.9	0.02
Rat, Whole Blood	7	1.7	0.062
Goat, Whole Blood	4.7	1.2	0.085

^a Initial velocity obtained from pooled blood or serum.

^b Experimental values obtained in 0.25 M., pH 7.4, 37° phosphate buffer.

^c Extrapolated.

^d Molarity of NaHCO₃ in 0.25 M. pH 7.4, 37° phosphate buffer required to produce a reaction rate similar to 100% biological medium.

To demonstrate the presence of a blood factor responsible for this enhanced activity, pooled mouse serum and pooled guinea pig serum were adjusted to pH 3 in separate experiments and evacuated for 30 minutes at 20 mm Hg to remove most of the dissolved carbon dioxide. The pH was returned to 7.4 with dilute sodium hydroxide solution, and the sera were evaluated. Both samples were essentially unreactive toward nor-HN₂. To each serum was added 0.025 M. sodium bicarbonate (approximate physiologic concentration) and the sera were again tested. The mouse serum reacted as might be expected in the absence of a rate acceleration factor and exhibited a rate of disappearance of nor-HN₂ consistent with Fig. 3. The reaction rate of the guinea pig serum, however, was approximately ten times greater than that of the mouse serum.

It appears, therefore, that the reaction between nor-HN2 and blood serum does not occur in the absence of carbon dioxide, but is accelerated beyond its normal rate in the presence of certain mammalian sera. Furthermore it appears that the factor which promotes the rapid reaction between nor-HN2 and blood serum is not inactivated when held at pH 3 for 30 minutes. This factor has been observed in the whole blood or serum of humans, goats, dogs, rabbits, rats and guinea pigs; muscle tissue from rabbits, rats, and rat liver. The concentration of the factor in blood varies not only between species but between animals of the same species.

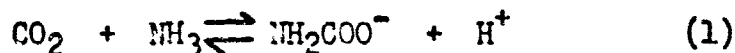
B. Mechanism of the Transformation of Nor-HN2 in Aqueous Bicarbonate and Blood

Measurement of the rate of disappearance of nor-HN2 by the MBP method showed that the pseudo first order rate constant of equation 2 increases in a linear manner with increasing concentration of carbon dioxide as shown in Figure 3.

Since the MBP method measures only the rate of disappearance of alkylating agent, additional evidence for the mechanism of the disappearance was sought. For this purpose the rate of formation of chloride ion was determined at 37° in 0.1 M., pH 7.4 phosphate buffer. In the range of concentration of 0.021 M. to 0.05 M. sodium bicarbonate, the rates of disappearance of nor-HN2 as measured by the MBP method agreed with the rates of formation of chloride ion (after correction for Cl^- due to Eq. 1). Thus, the rates of disappearance of nor-HN2 in Figure 3 depict also the rates of 3-(2-chloroethyl)-2-oxazolidinone formation.

The linearity of Figure 3 indicates second order kinetics. The rate controlling step in equation 2 is therefore the bimolecular step 1. In these studies, only the forward reaction of step 1 (Eq. 2) was considered since the backward process is hindered in basic media.^d

^d The reaction between ammonia and carbon dioxide has been studied by Pinsent and co-workers (13). The reactions involved are:



All the hydrogen ions produced in reaction (1) are instantly transformed into ammonium ions by equation (2) and the overall rate is governed by the speed of reaction (1), i.e.,

$$-\frac{d[\text{CO}_2]}{dt} = k [\text{CO}_2] [\text{NH}_3]$$

The value of the velocity constant k was found to be $1130 \text{ M}^{-1} \text{ Sec}^{-1}$ at 40°.

In order to accelerate the overall rate of equation 2 the active blood factor must alter the rate controlling step (or both steps). Since the rate controlling step of equation 2 is carbamate ion formation (step 1) it appears that the function of the blood factor is to accelerate this step.

One could logically suppose that the role of the blood factor is to increase the availability of carbon dioxide in accordance with the following equation:



Equation 4 is catalyzed by the enzyme carbonic anhydrase (14). The secondary nitrogen mustards might therefore be expected to react more rapidly in the presence of this enzyme. To test this hypothesis, carbonic anhydrase (at various concentrations) was added to the pH 7.4 buffered system containing nor-HM2 and sodium bicarbonate. No acceleration in the reaction rate was observed. Furthermore, the reaction of nor-HM2 in fresh guinea pig blood serum proceeded undisturbed in the presence of 10^{-2} molar sodium cyanide, a concentration sufficient to cause serious inhibition of carbonic anhydrase (15). It is concluded that carbonic anhydrase plays little or no part in accelerating the reaction rate.

C. Properties of the Factor

Thermal Inactivation

To determine the thermal stability of the factor, the carbon dioxide was removed from guinea pig serum as above and the serum was heated to 75° for 5 min. Upon the addition of 0.025 M sodium bicarbonate and reaction with nor-HM2, a rate of disappearance approximating that shown in Fig. 3 was observed. The blood factor that promotes the reaction between nor-HM2 and blood serum appears to have been destroyed by this treatment. It is, however, quite stable at lower temperatures. Heating to 50° for 5 days did not destroy the activity. Thus, this substance exhibits an extremely high temperature coefficient of thermal inactivation, a characteristic of proteinaceous material. The active factor is normally stored as lyophilized serum at refrigerator temperatures.

Dialysis

The factor described herein is not dialyzable. A sample of guinea pig serum was dialyzed against running tap water for 18 hours, and the carbon dioxide was removed by treatment under the acidic conditions described above. Upon the addition of 0.025 M sodium bicarbonate, the original high activity of the serum was restored.

D. Optimum pH

The rate of reaction between fresh goat blood and nor-HN2 at 37° versus pH is plotted in Fig. 4. The pattern of variation in reaction rate as a function of pH illustrates an optimum pH (7.2). It is noteworthy, however, that upon decreasing the pH below the pK_a of nor-HN2 (6.5) the decrease in reaction rate is more abrupt; suggesting that the optimum pH shown in Fig. 4 is altered due to protonation of the substrate, nor-HN2, at the lower pH values.

Due to the unavailability of free CO_2 at high pH, the reaction of nor-HN2 in the presence of sodium bicarbonate would be expected to proceed more slowly as the pH is increased. The cyclization process is pH dependent and proceeds more rapidly at high pH. Both processes probably contribute to the steep slope of Fig. 4 at high pH.

E. Interpretation

The data presented reveals the presence of an ingredient in blood serum that is capable of increasing the reaction rate between certain secondary nitrogen mustards and carbon dioxide. Although this substance does not react directly with nor-HN2, it causes an accelerated loss of nor-HN2 in the presence of carbon dioxide.

This active principle of blood is evidently an enzyme but is not carbonic anhydrase, is not inhibited by 10^{-2} M cyanide ion, and is non-dialyzable. It is not inactivated when held at pH 3 for 30 min. although gradual inactivation occurs when allowed to stand at this pH for longer periods of time. The active principle is destroyed when heated to 75° for 5 minutes and exhibits an extremely large temperature coefficient of thermal inactivation. The blood level of the factor varies from animal to animal and between species.

The reaction of certain secondary nitrogen mustards with carbon dioxide converts them rapidly to compounds that are not potent as alkylating agents and whose pharmacologic properties have not been investigated. When these mustards are employed as cancer chemotherapeutic agents, or in other in vivo biological studies, most of the injected compound will be immediately transformed to another chemical compound. Caution must therefore be exercised in predicting and interpreting the in vivo activity of such mustards.

F. Implications in Respiration

The in vivo formation of carbamate ion is of considerable biologic importance and has been shown to be involved in the respiratory processes (16). A significant fraction of the total carbon dioxide in the blood is transported in the form of carbamate

ion through the plasma proteins and hemoglobin (16).

Since carbamate formation is a nucleophilic reaction, free unprotonated amine is required. The reaction between amines of high basicity, (i.e., high relative to physiologic pH) and carbon dioxide is rapid when conducted in highly basic media (10). When conducted at lower pH levels (closer to pH 7.4), however, such amines are protonated and essentially no reaction can occur. Thus any carbamates formed from highly basic amines and carbon dioxide should be stable and not readily reversible in areas of low CO_2 partial pressure.

Only those amines of relatively low pK_a are suitable for the formation of carbamates capable of releasing carbon dioxide in the lungs. These are not highly protonated at physiologic pH and would be expected to react with carbon dioxide. Although it is not normally present in biological systems, nor- H_2 is an example of such an amine, and its reaction rate with carbon dioxide at pH 7.4 is measured in minutes. Blood is exposed to the lowered partial pressure of carbon dioxide in the lungs for only approximately one second (17). The unassisted rapid turnover of carbon dioxide by chemical means alone (via carbamate formation) is improbable in this short period of time.

It is postulated herein that an enzyme is present in blood serum that is capable of accelerating the rate of carbamate ion formation (as depicted in Step 1 of Equation 2). That this ingredient should possess so high a specificity as to accelerate carbamate ion formation of only certain secondary nitrogen mustards is unlikely. Presently, however, we have no evidence to indicate that non-mustard amines such as hemoglobin undergo catalysis by this enzyme to form carbamate ion.

Although amines react with CO_2 to form carbamate ion, the instability and reversibility of this compound has delayed the clear demonstration of an enzyme that catalyzes this process. The discovery of the trapping function through cyclization of a 2-chloroethylamine group to form a stable oxazolidinone (Equation 2) has enabled us to demonstrate an enzyme that catalyzes the 1st step of the reaction. It is probable that the action of this enzyme is not limited to 2-chloroethylamines but operates with other naturally occurring amines as well and thereby serves an important function in CO_2 transport in the body.

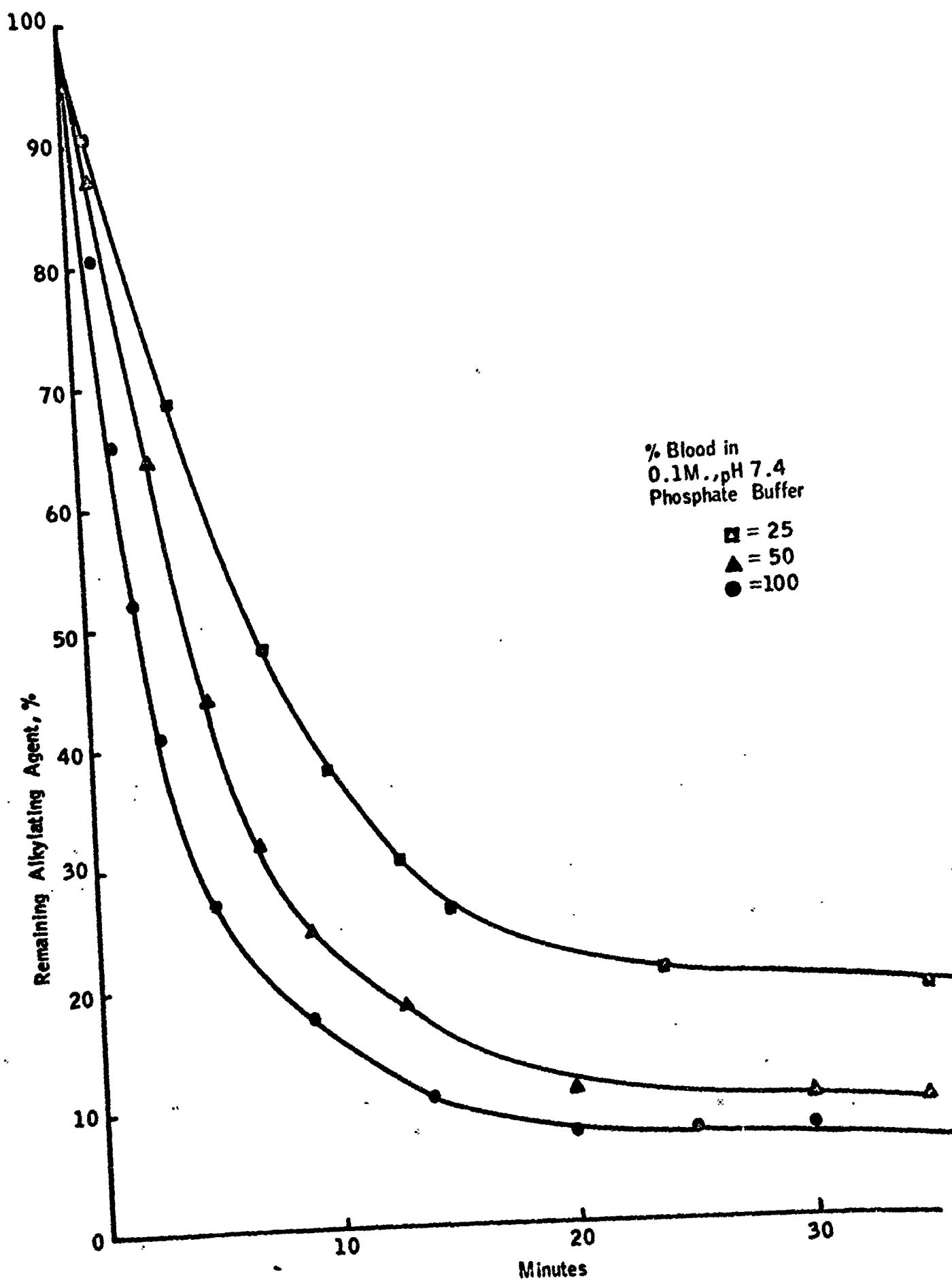
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Fig. 1. Variation in reaction rate of nor-EN2 as a function of concentration of fresh rabbit blood. Blood dissolved in 0.1 M., pH 7.4 phosphate buffer. Reaction maintained at pH 7.4, 37°. Concentration of nor-EN2 = 50 µg/ml.

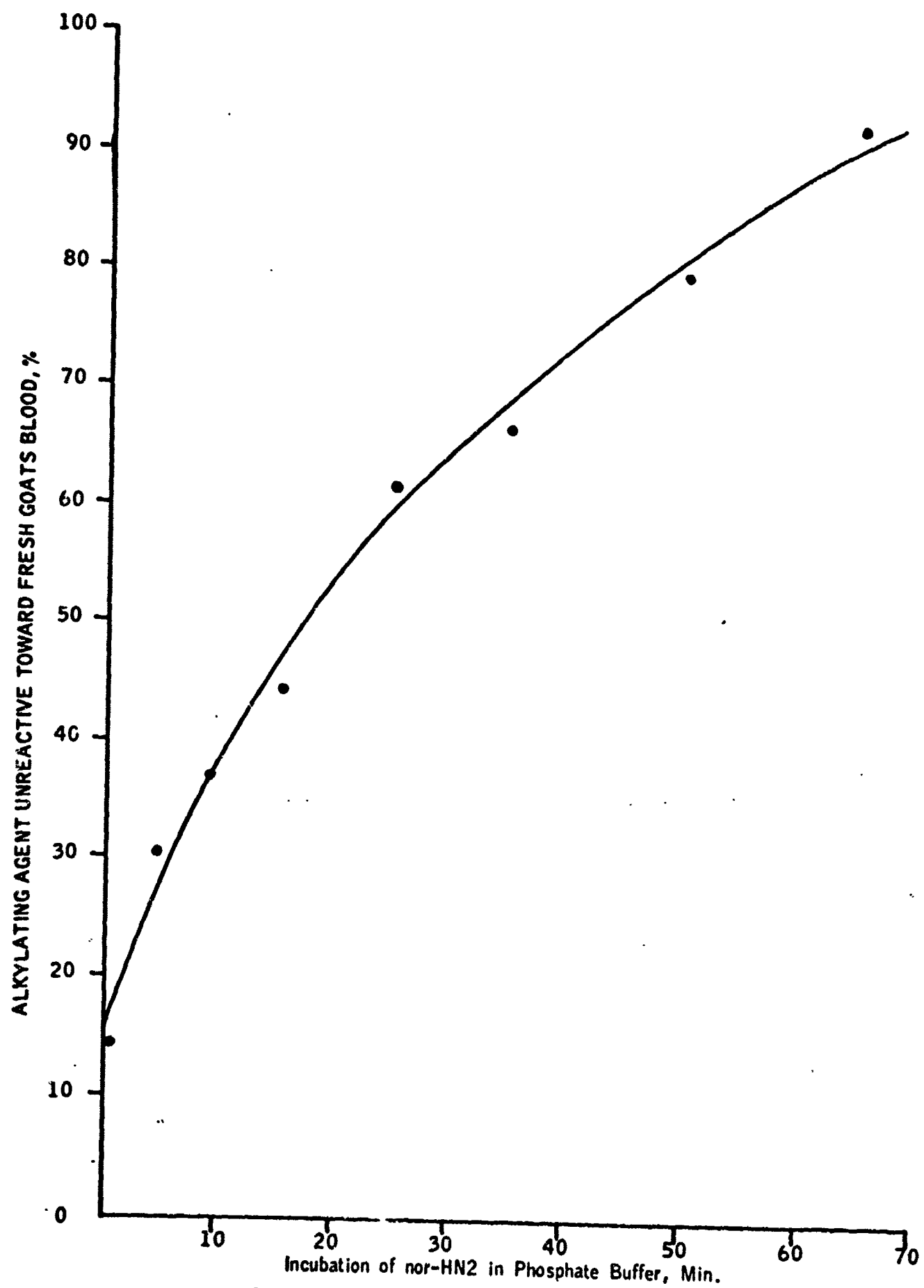
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.. Fig. 2. Inability of fresh goat blood to destroy precyclized nor-MN₂. Plot of percentage of alkylating activity unreactive towards fresh goat blood after incubation in 0.1 M, pH 7.4 phosphate buffer for various periods of time at 37°. Concentration of nor-MN₂ = 50 µg/ml.

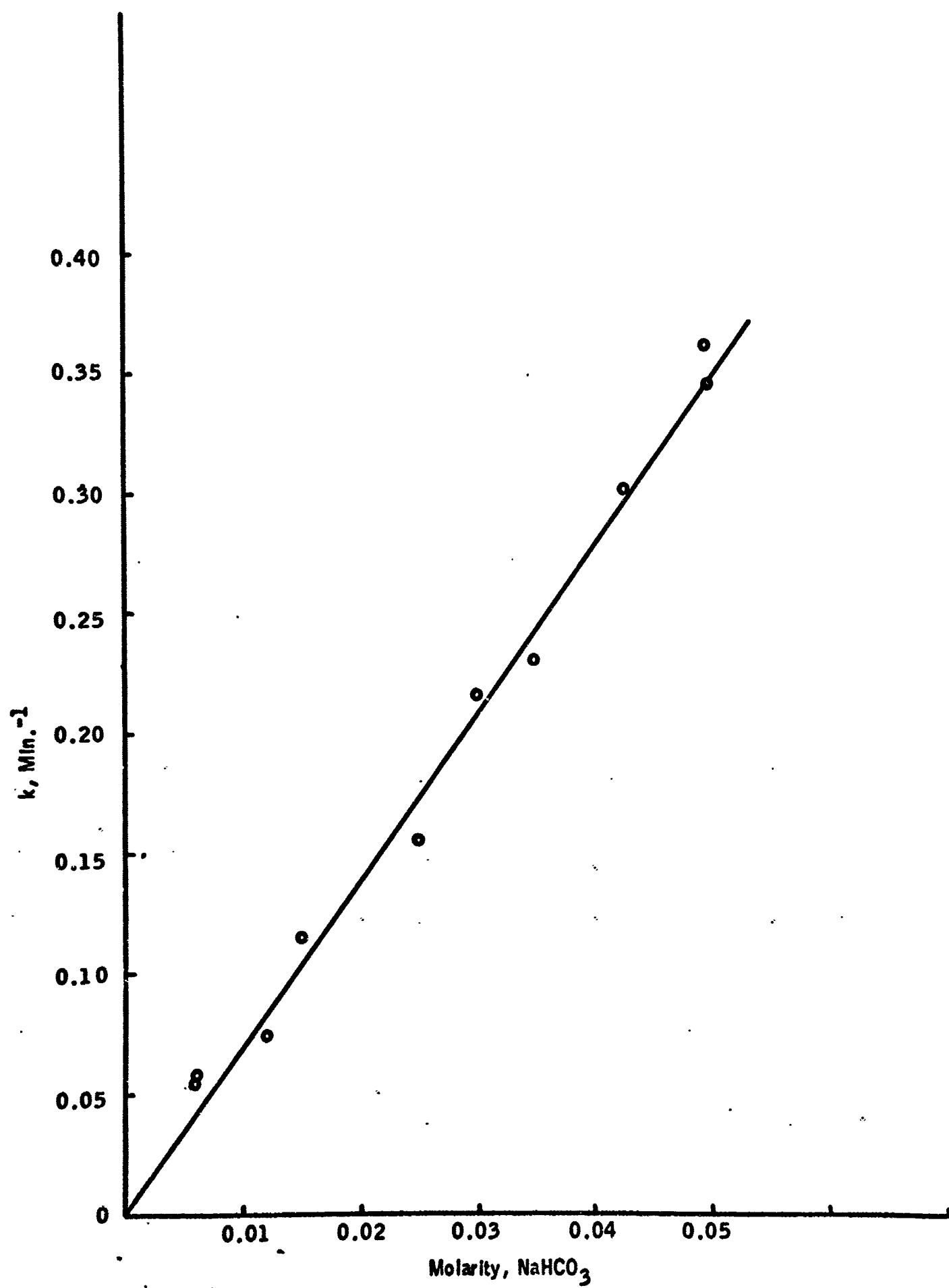
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Fig. 3. Reaction of Nor- F^{12} (75 $\mu\text{g}/\text{ml.}$) with Sodium Bicarbonate in 0.25 M., pH 7.4 Phosphate Buffer, 37°. Pseudo First Order Rate Constants versus Molarity of Bicarbonate.

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Fig. 4. Variation of the reaction rate of nor- H_2 and freshly drawn goat blood as a function of pH, 37° .

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